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New Tool for Studying Interactions of Components of Ribonucleic Acid Polymerase: Rifampin-Dependent Mutants

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Mutants of *Escherichia coli* showing a rifampin-dependent phenotype were isolated from cells of strain CP78 mutagenized with ethyl methane sulfonate or nitrosoguanidine when an antibiotic underlay technique was used. The mutants varied greatly in their rifampin requirement. The minimum necessary concentration ranged from 1 to 50 μg/ml. The mutants could be divided into four phenotypic classes. These dependent mutants and their revertants should be a useful tool for probing interactions between the component polypeptides of ribonucleic acid polymerase and for studying the linkage of transcription with other cellular processes.

Rifampin is a specific inhibitor of DNA-dependent RNA polymerase (for a review, see reference 15). In *Escherichia coli* this enzyme is a multimer consisting of several subunits, and in the holoenzyme the stoichiometry is $\alpha_2\ \beta\ \beta'\ \sigma$ (16). Rifampin-resistant mutants, *rpoB*(R), have an altered β subunit (10), and reconstitution experiments have shown that the β subunit is the site of inactivation by rifampin.

A number of factors may influence the activity of DNA-dependent RNA polymerases. These include termination factor ρ (11), factor ψ (13), and the CAP protein (17). There may also be linkage between transcription and translation which could take the form of a protein or other molecule that modulates the activity of RNA polymerase and ribosomes. Candidates to fill such a role have been described, namely, protein synthesis factor Tu, Ts (12), and $tRNA_f^{Met}$ (9). An approach to a possible link between transcription and translation is through in vivo studies with mutants. In some such studies (1, 2) a ribosomal phenotype was modified by mutations conferring rifampin resistance.

The specific impetus that led to the attempt to isolate rifampin-dependent mutants was to reverse the just-described phenomenon: namely, could mutants be isolated in which the phenotype due to *rpoB*(R) mutations was altered by mutations in ribosomal components? Such mutants and their revertants would also be useful in studying interactions between the component subunits of RNA polymerase itself.

Direct spreading of cells onto plates containing rifampin did not lead to the isolation of antibiotic-dependent mutants, although various antibiotic concentrations, selection temperatures, and mutagens were used. Therefore, use was made of an antibiotic underlay technique. This brings the cells into contact with antibiotic more gradually and has been successful in isolating several interesting types of antibiotic-dependent mutants (3, 5, 6). Cells of strain CP78 mutagenized with ethyl methane sulfonate (EMS) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and introduced to antibiotic by the antibiotic underlay technique gave rise to mutants some of which were rifampin-dependent (Rfm^d). [The term Rif^d, which might otherwise be employed by analogy with Rif^r, has been avoided since *rif*^{d} has already been used to describe *rpoB*(R) mutations that are dominant to *rpoB*(S) in heterodiploids (8)].

The selection of Rfm^d mutants is shown in Table 1. The effect of temperature on the frequency of dependent mutants was different, depending on whether the cells were treated with EMS or NTG. With NTG, the fraction of mutants growing up which had a Rfm^d phenotype was similar whatever the selection temperature. With EMS-treated cells, the fraction of mutants that were Rfm^d strongly correlated with the temperature of the selection. This suggested that there was an underlying difference in the mutants produced by the two mutagens. However, there was no significant difference in phenotype (with respect to different antibiotic concentrations and different temperatures) of mutants from EMS- or NTG-treated cells. About 30 rifampin-dependent mutants were isolated from CP78. They were designated PB1-32.

Mutant growth at rifampin concentrations between 1 and 400 μg of antibiotic per ml was tested by spotting onto plates. The minimum concentration for sustained growth varied very widely from as little as 1 μl (PB24) to about 50

μg (PB1) of rifampin per ml. All mutants grew well in the presence of 100 to 400 μg of rifampin per ml. Most mutants grew optimally with a generation time in liquid rich medium (4) of 60 to 90 min, three to four times slower than CP78. The range was from 40 min (PB25) to 180 min (PB5).

TABLE 1. *Isolation of rifampin-dependent mutants*

Mutagen	Rfm[d] colonies			% Rfm[d]		
	30°C	37°C	42°C	30°C	37°C	42°C
EMS	0/810[a]	4/792	10/916	0.0	0.5	1.1
NTG	5/421	5/358	3/404	1.2	1.4	0.7

[a] Fraction of colonies from those isolated at a particular selection temperature that show a Rfm[d] phenotype at one or more temperatures (30, 37, 42°C). Strain CP78 *thi-1 thr-1 leu-6 his-65 arg-46 gal-3 xyl-7 malA1 mtl-2 aro-13* F⁻ was used in this selection. EMS and NTG mutageneses were done as described in reference 5. For selection of mutants, an antibiotic underlay technique was employed, essentially as described previously (5). Mutagenized cells were spread on plates containing about 30 ml of medium and incubated for 3 h at 37°C. Plates were then underlaid with 0.15 ml of a stock solution of 40 mg of rifampin per ml. The final concentration of rifampin in the plates was therefore about 200 μg/ml. After underlaying with antibiotic, plates were stored at 4°C for 28 to 36 h. They were then further incubated at 30, 37, or 42°C for about 72 h (37, 42°C) or 100 to 120 h (30°C). Colonies appearing were streaked for single colonies on plates containing 200 μg of rifampin per ml. The total number of colonies growing up was similar at all temperatures. About twice as many colonies were obtained on plates with EMS-mutagenized cells, compared with plates with NTG-mutagenized cells. Under the conditions used in these experiments, killing was 10 to 20% in the case of EMS and 40 to 60% in the case of NTG.

The mutants were classified on the basis of their antibiotic response at different temperatures and most fell into one of four classes. The first class was Rfm[d] at all temperatures (e.g., PB11; see Table 2). The second class was Rfm[d] at lower temperatures (37°C and below) but antibiotic indifferent at higher temperatures (e.g., PB4). The third class was Rfm[d] at 30 and 37°C and did not grow at 42°C (e.g., PB2). The fourth class was Rfm[d] at 37 and 42°C, but did not grow at 30°C (e.g., PB9).

Classes I and III were most often found (about 40 and 30% of the mutants, respectively), but the proportion of each type seemed quite strain dependent: a selection with *E. coli* B strain L44 gave rise to mutants, 40% of which fell into class II.

The dependent phenotype was quite stable, but by plating cells in the absence of antibiotic, antibiotic-independent revertants could be obtained from all mutants.

For most mutants, the frequency of reversion lay in the range 10^{-3} to 10^{-4} (all determined at 37°C), although in several cases (PB1, 9, 12, 17, 18) the frequency was about 10^{-5}. The reversion frequency of selected mutants is included in Table 2.

Characterization of the mutations in these strains responsible for the phenotype is in progress. Preliminary genetic analysis of five mutants by P1 transduction indicated that a *rpoB*(R) mutation was always present and separable from

TABLE 2. *Some characteristics of selected rifampin-dependent mutants*

Mutant strain	Selective conditions		Phenotype												Reversion frequency[b]
	Mutagen	Temp (°C)	30°C				37°C				42°C				
			0	3.2	32	200[a]	0	3.2	32	200	0	3.2	32	200	
PB2	NTG	37		+++	+++	++[c]		+++	+++	+++					10^{-4}
PB4	NTG	37			++	++			+++	+++	+++	+++	+++	+++	10^{-4}
PB5	EMS	37						+	+	+			±		10^{-3}
PB9	NTG	42								+++				+++	10^{-5}
PB11	EMS	42			+++	+++			+++	+++				+++	10^{-4}
PB12	EMS	42						+	+	+++				±	10^{-5}
PB28	NTG	42			++		+++	+++	+++	+++	+++	+	±		10^{-3}

[a] Micrograms of rifampin per milliliter.

[b] There was a variable amount of residual growth after plating cells in the absence of exogenous rifampin. The frequency of production of rifampin-independent revertants was rounded to the nearest order of magnitude. All values are for 37°C, except PB28 (30°C). Revertants tested showed variable resistance, but were more resistant than parental strain CP78. The antibiotic phenotype was determined by spotting on plates containing 1, 1.7, 3.2, 5.6, 10, 17, 32, 56, 100, 200, and 400 μg of rifampin per ml. For rifampin stock solutions, used in underlaying and in plate preparation, the antibiotic was dissolved in methanol at 100× the final concentration desired. Rifampin was obtained from Boehringer GmbH, Mannheim.

[c] Cultures were spotted on rich plates containing the indicated concentration of rifampin. Growth was scored after 48 h (37, 42°C) and 72 h (30°C). + to +++ indicate poor to good growth; where nothing is indicated, there was no growth. Parental strain, CP78, can grow on 5.6 but not 10 μg of rifampin per ml (at 37 and 42°C). At 30°C, it can grow on 3.2 but not 5.6 μg of antibiotic per ml.

the mutation producing the dependent phenotype. So, the Rfm^d phenotype is the product of a *rpoB*(R) mutation plus additional mutation(s) conferring dependence. The location of additional lesions might be as follows: (i) also within the β subunit, (ii) within another subunit of the holoenzyme, (iii) in components that may modulate RNA polymerase activity such as CAP protein or $tRNA_f^{Met}$, or (iv) in some other cellular function not normally connected with the transcription process. Genetic analysis should help distinguish which of these possibilities is in fact the case. Two-dimensional polyacrylamide gels (7) of Rfm^d mutants have revealed several instances of alterations in ribosomal proteins. Whether these mutational alterations are involved in the phenotype is now being investigated.

In vitro studies of the RNA polymerase of these mutants would also be worthwhile. The stoichiometry of binding of rifampin to enzyme has been established to be 1:1 (14). The very great variation in the concentration of antibiotic necessary for the growth of Rfm^d mutants is not likely to be a reflection of an altered stoichiometry. More likely it reflects the degree to which rifampin has to remain bound for the polymerase to be active: there may be a range from cases in which it acts catalytically to alter enzyme conformation and is then released to cases in which rifampin has to remain bound to the enzyme for the polymerase to be active.

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